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Differential Expression of the *KIT* Gene in Liaoning Cashmere Goats with different Coat Colors

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Authors' Contributions
JL and QZ designed the experiment and wrote the manuscript. QJ and WC extracted RNAs and performed qRT-PCR. YL and HJ performed western blotting and immunohistochemistry staining. JH conducted statistical analysis.

Key words
***KIT*, Melanin, Liaoning Cashmere goat, Mutation, Immunohistochemical.**

ABSTRACT

KIT encodes a growth factor receptor that is expressed in the precursor of the melanophore. It plays an important role in the multiplication, migration and survival of melanophores. As of yet, no studies have addressed the diverse expression of the *KIT* gene and its protein in goats of different fur colors. The effect of *KIT* mutations on *KIT* protein expression was examined in white cashmere and black cashmere goats. A single A→G missense mutation in exon 13 differentiated cashmere goats with different colors. Only a histidine (H)→arginine (R) amino acid (AA) change was detected at *KIT* exon 13 in both the white cashmere goat and the black cashmere goat. Moreover, comparison with other species revealed three dramatic amino acid mutation areas. Our results also indicated that c-kit expression was higher in the white cashmere goat than in the black goat, and this significant difference was detected by q-PCR and western blotting. All cashmere goats of different colors examined by immunohistochemical analysis showed either weak (the black cashmere goat) or strong (the white cashmere goat) expression of the *KIT* protein. These findings suggested a relationship between mutations in *KIT* exon 13 and differential fur color in cashmere goats. These results lay the foundation for further research on exon 13 of the *KIT* gene and color regulation in cashmere goats.

INTRODUCTION

Coat color is one of the most important breeding traits in horses, goats and other domestic animals (Fontanesi *et al*., 2011). Among fiber-producing animals, the new breed of Liaoning goats that produce cashmere, also known as "fiber gem", possesses qualities such as high cashmere yield and good cashmere fineness (Kambe *et al*., 2011). Studies have identified a number of genes that regulate the fur color of cashmere goats. *KIT* is a type III receptor tyrosine kinase that binds to the ligand *MGF* and plays a crucial role in the growth and differentiation of melanocytes, hematopoietic cells, and germ cells. The *KIT* and *MGF* genes are associated with pigmentation disorders, anemia, sterility and recessive lethality (Besmer *et al*., 1993; Pawson and Bernstien, 1990). Mutations at the *KIT* locus can lead to pleiotropic developmental defects in pigment cells, and *KIT* activation impacts blood cells (Geissler *et al*., 1988; Haase *et al*., 2010). For instance, *KIT* mutations cause dominant white spotting (*KIT*W) in mice and piebaldism in humans, which both display strikingly similar white patches of hair and skin in heterozygous individuals (Ezoe *et al*., 1995; Geissler *et al*., 1988). *KIT* also plays a pivotal role in melanocyte migration, development and proliferation (Grichnik, 2006). Studies have confirmed the presence of the semidominant IP allele and the dominant I allele in the pig, which are both associated with a duplication of the *KIT* gene (Johansson-Moller *et al*., 1996). The white color and the mode of inheritance are controlled by an autosomal dominant allele designated I (*KIT*) for 'inhibition of

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color' (Ollivier and Sellier, 1982). The I allele in pigs leads to a complete loss of skin pigmentation. In contrast to murine *KIT* mutants, homozygous I/I pigs are fully fertile (Marklund *et al*., 1998). In addition, murine *KIT* mutations are often homozygous lethal or sublethal. Dominant white (I) pigs lack mature melanocytes in the skin as well as lacking precursor melanocytes as would be anticipated for a *KIT* mutation (Johansson-Moller *et al*., 1996). Hence, *KIT* expression is related to the prevention of severe pleiotropic effects on other tissues caused by the gene duplication in *I/I* (*KIT*). We report the differential expression of *KIT* in dominant white and black cashmere goats at the gene, protein, tissue and epigenetic levels. The results indicate that three mutation areas exist in exon 13 of *KIT*, and one of them (159-171 AA) influences the coat color of cashmere goats. Using immunohistochemical analysis, we uncovered the distribution of c-*KIT* in the skin of cashmere goats. In this study, *KIT* was selected to investigate the association of polymorphisms with fur color in the cashmere goat.

MATERIALS AND METHODS

Ethics statement

Animal experiments were conducted in strict accordance with the guidance for the care and use of laboratory animals by the Jilin University Animal Care and Use Committee (permit number: SYXK (Ji) 2008-0010/0011). All production traits were measured with standardized methods.

Sample collection and RNA extraction

Cashmere goats were acquired from the BaiShang Livestock farm in Changchun, China. The cashmere goats were classified into two groups according to their black or white fur color. A section 5.0 cm in diameter was sheared in the shoulder blade of the goats. The samples were first disinfected in ethanol followed by placement in Hanks solution and taken to the laboratory where they were kept at -80°C.

To extract total RNA from the skin, each sample was frozen in liquid nitrogen and ground. The sample was placed into a centrifuge tube containing 1 mL TRIzol reagent and incubated for 5 min followed by the addition of 200 µl chloroform and mixing. The sample was incubated for 10 min and centrifuged at 12000 g for 15 min at 4°C. The supernatant was collected in a clean centrifuge tube, and 500 µl isopropanol was added and mixed, incubated for 10 min, and centrifuged at 12000 g for 10 min at 4°C. After centrifugation, the supernatant was removed. The sample was washed several times with 1 mL cold 75% ethanol and centrifuged at 7500 g for 5 min at 4°C, and the supernatant was discarded. After drying for several minutes, 20 µl DEPC-treated water was added, and the samples were kept at -80°C.

The analysis of RT-PCR and cDNA sequencing

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RT-PCR was performed for *KIT* gene exon 13 using an ImProm-II RT system (Promega) according to the manufacturer's instructions. Primers were designed using Primer 5.0 and synthesized by AuGCT Biotechnology Co. The primers were *KIT*-exon13 (5'-GGYAATCACATGAATAATGTGAA-3') for the forward reaction and *KIT*-exon13 (5'-TCACCATAGCAACAATATTCTGT-3') for the reverse reaction (GenBank accession MGI: D45168.1). PCR amplifications were performed with a 5 min pre-incubation at 94°C followed by 35 cycles of 30 s at 94°C and 30 s at 60°C. PCR products were verified by melting curve analysis, agarose gel electrophoresis, gel purification and DNA sequencing.

Real-time quantitative reverse transcription-PCR

For real-time quantitative reverse transcription (RT)-PCR, total RNA was reverse transcribed using an ImProm-II RT system (Promega) according to the manufacturer's instructions. For detection and quantification, a MyiQ real-time PCR detection system (Bio-Rad) was used. PCRs were performed using a SYBR Premix Ex Taq II (Takara, Seoul, Republic of Korea). PCRs were carried out in a final volume of 20 mL using 0.5 mM of each primer, cDNA, and 10 mL of the supplied enzyme mixture containing the DNA double-strand-specific SYBR Green I dye for detection of PCR products. PCRs were performed with a 3 min pre-incubation at 95°C followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. PCR products were verified by melting curve analysis and agarose gel electrophoresis. The standard curve was exported from the MyiQ real-time PCR detection system (Bio-Rad). The difference in efficiencies was less than 0.1, indicating similar amplification efficiencies of the two cDNAs. The relative amount of mRNA to GAPDH RNA was calculated using the equation $2^{-\Delta\Delta CT}$ where $\Delta\Delta CT = (\Delta CT\ mRNA - \Delta CT\ GAPDH)$ (Yuan *et al*., 2006).

Western blotting analysis

Total protein from skin samples from differently colored goats was extracted with Thermo Scientific M-PER Mammalian Protein Extraction Reagent. The protein concentrations were determined using a BCA™ protein assay *KIT*. Aliquots of the lysates were separated

on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) with a glycine transfer buffer [192 mM glycine, 25 mM Tris–HCl (pH 8.8), 20% methanol (v/v)]. After blocking nonspecific sites with blocking solution [5% (wt/vol) nonfat dry milk], the membrane was incubated overnight with a specific primary antibody at 4°C. The membrane was then incubated for an additional 60 min with a peroxidase-conjugated secondary antibody at room temperature. The immuno-active proteins were detected using an enhanced chemiluminescence (ECL) western blotting detection *KIT* (Chen *et al*., 2013).

Immunohistochemistry

Epidermis samples from white and black cashmere goats were fixed in formalin-buffered saline and embedded in paraffin. Tissue sections (5 mm) were deparaffinized in xylene for 10 min, dehydrated in alcohol, and rinsed with PBS. For exposure and detection of the *KIT* protein, antigen retrieval was performed by heating in the microwave in citric acid-sodium citrate for 10 min. Nonspecific binding was blocked by incubating the sections in 10% normal goat serum in Tris-buffered saline for 60 min. We used a rabbit antibody directed against human c-*KIT* (R&D Systems) at a concentration of 15 µg/ml. Binding was detected using an Cy3-goat-anti-rabbit IgG at a dilution of 1:100 for 40 min. Sections were washed in PBS and subsequently counterstained using DAPI (Haase *et al*., 2007).

Statistical analysis

Data are presented as the mean ± SD. Comparison between groups was made with a one-way analysis of variance (ANOVA; Dunnett's t-test) and Student's t-test. P-Values of 0.05 or less were considered statistically significant.

RESULTS

Sequencing analysis

The sequencing analysis indicates that *KIT* exon 13 has a total length of approximately 1000 bp excluding any introns. According to NCBI, it can translate approximately 333 amino acid residues. A prediction of the secondary structure of the protein which using DNAMAN7.0 coded by *KIT* exon 13 revealed a random coil, an α-helix and two transmembrane domains (Fig. 1C-II, 1C-III). The similarity of *KIT* exon 13 between the white and black goats was found to be up to 99% using DNAMAN7.0 to compare the predicted amino acid sequences. We screened exon 13 of the *KIT* gene in the dominant white goat and black goat, and identified a single-base A→G point missense

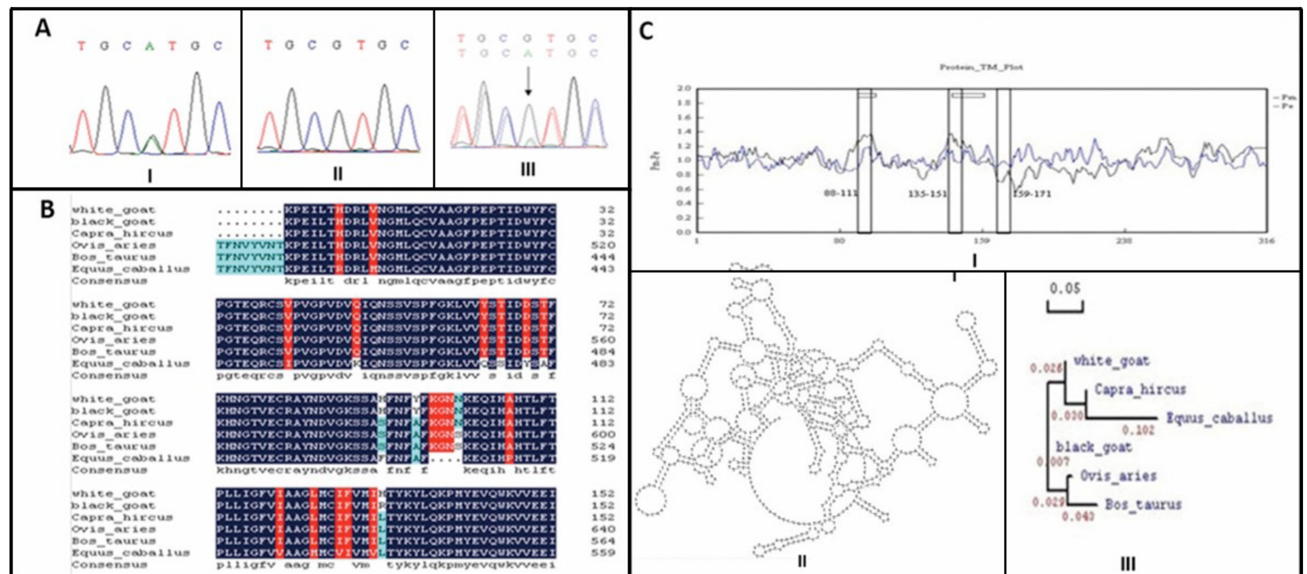

Fig. 1. A, The single mutation of KIT-exon13 in cashmere goats with different coat color. (I) Sequencing of KIT-exon13 in dominant white goat. (II) Sequencing of KIT-exon13 in dominant black goat. (III) Case showing nt 491 A→G missense mutation in white and black goat; B, Alignment of KIT-exon13 amino acid sequence prediction of cashmere goats with other mammals; C, The prediction of secondary structure in white cashmere goat and black cashmere goat. (I) The prediction of goat KIT-exon13 amio acid at the hydrophobic and the secondary structure. The unit of abscissa is amino acids digits and the ordinate is the value of the hydrophilic and hydrophbic balance(HLB). (II) The prediction of base secondary structure in goats. (III) Phylogenetic tree of KIT-exon13 gene of goats with different coat color.

mutation in all the samples (Fig. 1A). Only a single histidine (H)→arginine (R) amino acid (AA) difference was detected in exon 13 between the white and black goats. The change was found in the 159-171 aa area, which is in the second signal transduction extracellular transmembrane domain (Fig. 1B, 1C-I). Moreover, comparison with other species revealed that homology between the white cashmere goat and *Capra hircus* was 98%, *Equus* caballus was 70%, *Ovis aries* was 85% and *Bos taurus* was 75% (Table I). In goats, amino acid substitutions were detected in the exon 13 coding region of the *KIT* gene, namely, glutamine (Q)→lysine (K) between residues 88-111, tyrosine (Y)→alanine (A) between residues 135-151 and others (Fig. 1B). In addition, both mutations were in transmembrane domains. One mutation is located in the first transmembrane domain and the other on the second transmembrane domain (Fig. 1C-I). Phylogenetic tree analysis revealed that the white cashmere goat has the tightest genetic relationship with the black cashmere goat, comparatively stronger than that between *Capra hircus* and *Ovis aries* (Fig. 1C-III).

Analysis of expression level of KIT exon 13 by qPCR

Real-time RT-PCR analysis of *KIT* exon 13 mRNA from the skin of goats with different coat colors is shown in Figure 2A. The mRNA expression of *KIT* exon 13 analyzed by ΔΔCT was higher in the skin of white goats than in those with black coats (>6.31-fold) (Fig. 2A). This difference in *KIT* exon 13 mRNA abundance was significant ($p<0.01$) when assessed by one-way ANOVA. The qRT-PCR results confirmed that the expression of *KIT* in the white cashmere goat is higher than in the black cashmere goat.

Table I.- Comparison of the homology of goat KIT-exon13 gene with other mammals at the nt and aa levels (%).

KIT-13	*C. hircus*	*O. aries*	*B. taurus*	*E. caballus*
Amion Acid	98	85	75	70
cDNA	99	97	85	84

Western blot analysis of KIT protein expression

We performed a western blot on protein extracts from skin samples of a dominant white goat and a black goat. Our results displayed a strong band of the expected size (~145 kDa) for the full-length c-*KIT* protein. The black goat yielded a weak band at ~145 kDa.

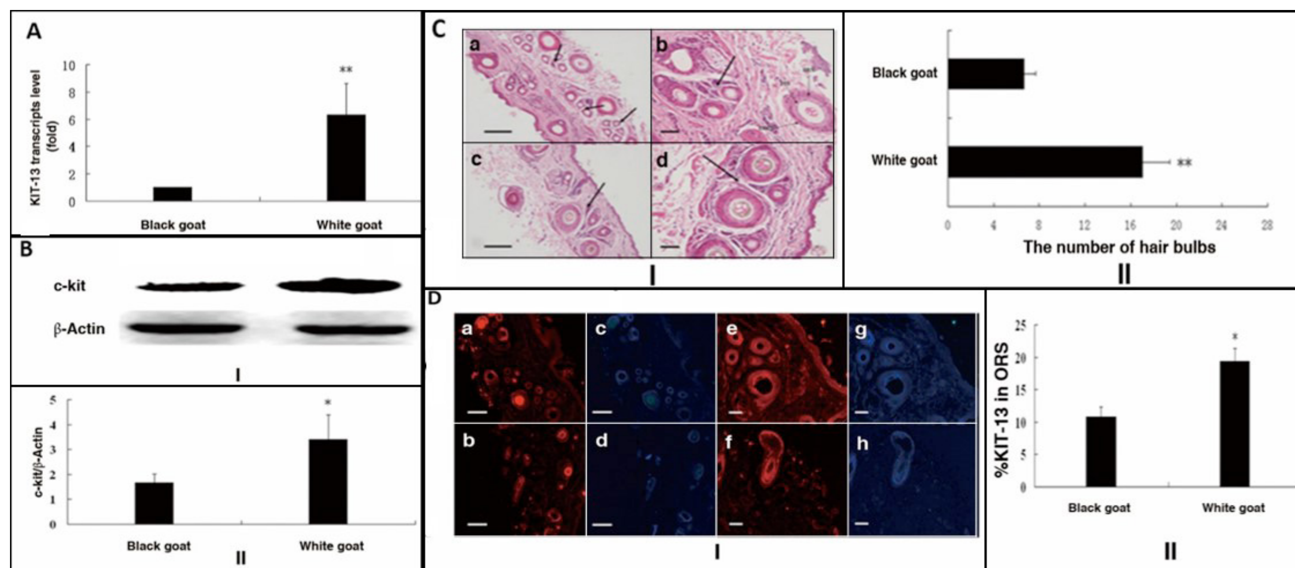

Fig. 2. A, Quantifications of *KIT*-exon13 in white cashmere goats and black cashmere goats; B, Western Blot analysis of c-*KIT* protein expression in skin samples with different coat color; **C,** H&E staining analysis of the expression of *KIT*-13 in skin samples about fair follicle and hair bulb for goats with different coat color. (a and c) the white goats and the black goats *KIT*-exon 13 expression (100×). (b and d) the white goats and the black goats *KIT*-exon 13 expression (200×). The number of hair bulbs analysis of c-*KIT* expression in skin samples with different coat color. ORS, outer root sheath; IRS, inner root sheath; HS, hair shaft cells; **D, (I)** Representative immunohistochemical staining of *KIT* in fair follicle for cashmere goats with different coat color. (a and b) the white goat and the black goat *KIT*-exon 13 anbidy expression (100×). (c and d) the white goat and the black goat *KIT*-exon 13 nucleus expression (100×). (e and f) the white goat and the black goat *KIT*-exon 13 anbidy expression (200×). (g,h) the white goat and the black goat *KIT*-exon 13 nucleus expression (200×). (II) The percentage of *KIT*-13 in different coat color goats' ORS.

Moreover, the tyrosine kinase receptor *KIT* was approximately 1.67-fold more abundant than β-Actin in the black goats and 3.39-fold more abundant than β-Actin in the white goats (Fig. 2B). The protein was significantly more abundant in the white goat than in the black goat with $p \leq 0.05$. Furthermore, the western blot results were in agreement with the *KIT* q-PCR results.

Immunohistochemical expression of KIT

In the developing embryo, hair follicle morphogenesis is regulated by reciprocal epithelial and mesenchymal interactions that occur in almost all organs (Toyoshima *et al*., 2012). The hair follicle is divided into the outer root sheath (ORS), the inner root sheath (IRS) and the hair shaft cells (HS). According to the immunohistochemical expression of *KIT* in dominant white and black goats, *KIT* was mainly found within the upper outer root sheath (ORS) and comparatively less in the bulge/hair germ. The white cashmere goat showed an approximate 1.5-fold expression in the tyrosine kinase receptor *KIT* compared to the black goat (Fig. 2D) with $p \leq 0.05$. Moreover, H & E staining analysis revealed more hair bulbs in single hair follicles in the white goat than in the black goat (Fig. 2C). These results indicate that *KIT* plays an important role in hair bulb differentiation.

DISCUSSION

The composition of animal coat color is a complicated process that is mainly dictated by the pigment content of the hair. The directional regulation of coat color in fiber-producing animals has economic value. According to previous studies, approximately 80 genes affect coat color, and half of the functional proteins encoded by them are located in the melanosome (Hearing *et al*., 1991). Furthermore, recent research has shown that genes related to melanin mainly include *MC1R*, *ASIP* (agouti), *TYR*, *steel*, *KIT*, and *ACIH* (Rieder *et al*., 2000). Among them, both *KIT* and *TYR* belong to the tyrosine family, and *TYR* can induce an increase in melanin. However, *KIT* mutations block the normal migration of melanocyte precursors leading to the absence of melanocytes in the hair follicle and resulting in a white coat color (Sarangarajan and Boissy, 2001; Scherer and Kumar, 2010). Therefore, research on *KIT* is important for the regulation of coat color. Marklund *et al*. (1998) have reported that the mutation of *KIT* in domestic pigs resulted in a decrease of white blood cells in I/I homozygous pigs. However, the relationship between *KIT* mutations and different goat coat colors was unclear. We studied the influence of *KIT* exon 13 in goats with different coat colors from the gene, molecule, tissue and epigenetic levels. The mutation of this gene had important implications on the epigenetic traits of animals. A lesion in the tyrosinase/*KIT* gene resulted in pigment lacking in the melanocytes in the albino mouse (Mayer and Green, 1968). Sequencing of the *KIT* exon 13 revealed that only a single (A→G) missense mutation differentiated the dominant white goat from the black goat (Fig. 1A). This single nucleotide polymorphism (SNP) was significant in the directional regulation of coat color. We speculated that the mutation would lead to a lack of pigment in the goat melanocytes. The q-PCR results indicated that the mRNA expression of *KIT* exon 13 in the skin of goats with a white coat color was higher than in those with a black coat by ΔΔCT (>6.31-fold, $p<0.05$) (Fig. 2A). Furthermore, we studied the amino acid secondary structure of *KIT* exon 13 in the dominant white goat and in the black goat. We found that the peptide encoded by *KIT* exon 13 spanned approximately 333 residues and had two transmembrane domains, and the Pm-Pe value indicated that the peptide was hydrophilic. On the basis of this analysis, we speculated that there were three amino acid mutation areas (residues 88-111, 135-151 and 159-171) (Fig. 1C-I). The missense mutation in position 171 coding for the alkaline histidine (H) in the white goat to the acidic arginine (R) in the black goat was located in the hydrophobic region of the second extracellular signal transduction domain. Several lines of evidence have demonstrated that the SCF/c-*KIT* pathway is critical to melanocyte survival under homeostatic, stimulatory or pathogenic conditions, including ultraviolet B exposure and pigmentation disorders (Hachiya *et al*., 2001; Hattori *et al*., 2004). Our result confirmed that H→R changed the structure of the SCF/c-*KIT* signal reception region, blocking the signaling pathway and reducing the production of melanin. In addition, the two sequences that diverged from other species were both located in the transmembrane domain, which influences the distribution of *KIT* exon13 in the different species (Fig. 1B). A mutation in position 135 of exon 13 coding the polar tyrosine (Y) →nonpolar alanine (A) suggests an interspecies difference. Our phylogenetic analysis also revealed that the *white goat* has the highest genetic relationship with *Capra hircus*, which is closer in comparison than with *Equus caballus* (Fig. 1C-III). These results may suggest that *KIT* exon 13 is species-specific.

The gene encoding *c-KIT* was mapped to the white spotting (W) locus (Chabot *et al*., 1988; Geissler *et al*., 1988) and, with its ligand, found to play an important role in the development of hematopoietic cells, germ cells and melanocytes. Beyond that, recent research has shown that the transcription factor NFIB was an unanticipated coordinator of stem cell behavior, blocking *KIT* signaling and ultimately preventing precocious melanocyte stem cell differentiation in the NFIB-deficient niche (Chang *et*

al., 2013). To verify previous q-PCR results, we used a western blot to investigate the expression of the *KIT* protein in skin samples of a dominant white goat and a black goat. The western blot results indicated that the expression level in the white goat was higher than in the black goat (>2.01-fold, $p<0.01$) (Fig. 2B). The western blot result for c-*KIT* was in agreement with the q-PCR results. In conclusion, mRNA and protein expression of c-*KIT* was higher in the white goat than in the black goat. *KIT* also played an important role in the distribution of melanin with goats.

It was reported that Dermal papilla (DP) was located in the center of the hair bulb and the dermal papilla cell (DPC) could induce the regeneration of the follicle (Hardy and Vielkind, 1996). H and E staining of goat skin revealed that a single hair follicle contained many hair bulbs (Fig. 2C). The average number of hair bulbs in the white goat was higher than in the black goat. Therefore, *KIT* might promote the differentiation of the hair bulbs and the regeneration of the follicle. To better understand the distribution, the immunohistochemical images showed that *KIT* was widely expressed in the skin of white goats and black goats (Fig. 2D). The staining was mainly located in the upper ORS of the hair follicle. The ORS, which is composed of layers of unpigmented cells, mainly provided a place for the differentiation of the follicular stem cells (Alonso and Fuchs, 2003). This result suggests that *KIT* has a role in the development of hair follicle stem cells.

CONCLUSION

This study provides evidence for the likely causative mutation for the dominant white phenotype in goats. We have also discovered that c-*KIT* has an effect on melanocyte migration, development and proliferation in goats of different coat colors. The implications of these finding clearly suggest that *KIT* is a key molecule in the regulation of coat color in goats. However, *KIT* is a large gene, and the missense mutation on *KIT* exon 13 is not the only mutation that can lead to the white phenotype in goats. Other factors may influence the white phenotype in goats, but these require further research.

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Conflict of interest statement

The authors declare that there is no confict of interest that could be perceived as prejudicing the impartiality of the research reported.

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